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The Enterovirus Protease Inhibitor Rupintrivir Exerts Cross-Genotypic Anti-Norovirus Activity and Clears Cells from the Norovirus Replicon

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Potent and safe inhibitors of norovirus replication are needed for the treatment and prophylaxis of norovirus infections. We here report that the *in vitro* anti-norovirus activity of the protease inhibitor rupintrivir is extended to murine noroviruses and that rupintrivir clears human cells from their Norwalk replicon after only two passages of antiviral pressure. In addition, we demonstrate that rupintrivir inhibits the human norovirus (genogroup II [GII]) protease and further explain the inhibitory effect of the molecule by means of molecular modeling on the basis of the crystal structure of the Norwalk virus protease. The combination of rupintrivir with the RNA-dependent RNA polymerase inhibitors 2′-*C*-methylcytidine and favipiravir (T-705) resulted in a merely additive antiviral effect. The fact that rupintrivir is active against noroviruses belonging to genogroup I (Norwalk virus), genogroup V (murine norovirus), and the recombinant 3C-like protease of a GII norovirus suggests that the drug exerts cross-genotypic anti-norovirus activity and will thus most likely be effective against the clinically relevant human norovirus strains. The design of antiviral molecules targeting the norovirus protease could be a valuable approach for the treatment and/or prophylaxis of norovirus infections.

Human noroviruses are a major cause of food-borne illness, accountable for 50% of all-etiologies outbreaks of acute gastroenteritis (both in developing and developed countries) (1, 2). Outbreaks often occur in long-term-care facilities and hospitals where the elderly and immunocompromised can become severely ill. The vast spreading nature of this virus commonly results in hundreds of sick individuals in each setting and may lead to the closure of hospital wards (3, 4). Associations with chronic gastrointestinal problems and cases of chronic gastroenteritis caused by noroviruses are being increasingly recognized (5, 6). There is no vaccine or antiviral treatment available against norovirus infections. Rupintrivir (AG-7088) is a protease inhibitor originally developed for the treatment of human rhinovirus infections (7, 8). Activity has also been demonstrated against other picornaviruses as well as against coronaviruses and caliciviruses (9, 10). Human noroviruses belong to the *Caliciviridae* family, whose protease displays a chymotrypsin-like fold with a cysteine as the active-site nucleophile and shares similarities with the 3C protease of picornaviruses (11). Rupintrivir has recently been reported to inhibit the replication of the Norwalk virus replicon (a genogroup I [GI] human norovirus) (10).

We here confirm and further document the inhibitory effect of rupintrivir against the human norovirus (by demonstrating that the compound is able to clear cells from the replicon) and demonstrate that the drug is antivirally active against the murine norovirus (MNV), a virus belonging to another genogroup of the genus. We study the inhibitory effect of rupintrivir against a GII 3C-like protease (in an enzymatic assay) and explain at the structural level the potential interactions between the inhibitor and the enzyme. The fact that the compound inhibits noroviruses of different genogroups suggests that the compound exerts cross-genotypic anti-norovirus activity and will very likely also be effective against other genogroups of the genus *Norovirus*, for which no cell culture system is currently available.

MATERIALS AND METHODS

Cells, viruses, and compound. HG23 cells (Norwalk virus replicon-bearing Huh-7 origin) were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Ghent, Belgium) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 20 mM HEPES, 0.075 g/liter sodium bicarbonate, 100 U penicillin/ml, and 100 μg/ml streptomycin at 37°C and 1.25 mg/ml of Geneticin (G418; Life Technologies) in a humidified atmosphere of 5% CO_2. MNV (virus strain MNV-1.CW1) was propagated in RAW 264.7 cells grown in DMEM (Life Technologies, Ghent, Belgium) supplemented with 10% or 2% FBS, 2 mM L-glutamine, 20 mM HEPES, 0.075 g/liter sodium bicarbonate, 1 mM sodium pyruvate, 100 U penicillin/ml, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Rupintrivir was a kind gift of Pfizer (La Jolla, CA).

Antiviral assay with the Norwalk replicon. The inhibitory effect of rupintrivir on human norovirus replication was assessed by quantification of the levels of Norwalk replicon RNA and of the reference (housekeeping) gene β-actin mRNA (by quantitative reverse transcription-PCR [qRT-PCR]). To this end, HG23 cells (5,000 cells/well) were seeded in 96-well plates in complete DMEM without the selection marker G418. Following an incubation period of 24 h, a serial dilution of rupintrivir was added to the cultures. After 72 h of incubation, cell monolayers were washed with phosphate-buffered saline (PBS) and collected for quantification of RNA load by qRT-PCR. Intracellular RNA was extracted from cells using the cell-to-cDNA lysis buffer (Ambion; Life Technologies). For detection of Norwalk virus replicon RNA, forward (5′-CCG GCT ACC TGC CCA TTC-3′) and reverse (5′-CCA GAT CAT CCT GAT CGA CAA G-3′) primers and probe (5′-FAM-ACA TCG CAT CGA GCG AGC ACG TAC-TAMRA-3′; FAM is 6-carboxyfluorescein and TAMRA is 6-car-

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boxytetramethylrhodamine) for the neomycin gene were used, as previously described (12). For detection of β-actin mRNA, forward (5′-GGC ATC CAC GAA ACT ACC TT-3′) and reverse (5′-AGC ACT GTG TTG GCG TAC AG-3′) primers and probe (5′-HEX-ATC ATG AAG TGT GAC GTG GAC ATC CG-BHQ1–3′) were used, as previously described (13). One-step qRT-PCR was performed in a 25-μl reaction mixture containing 12.5 μl One-Step reverse transcriptase qPCR master mix plus low ROX (Eurogentec, Belgium), 0.125 μl of RT-PCR enzyme mix, 5 μl of template RNA, and either 300 nM Norwalk replicon primers and probe or 300 nM β-actin primers and 200 nM probe. Cycling conditions were reverse transcription at 48°C for 30 min, initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min (ABI 7500 Fast real-time PCR system; Applied Biosystems, USA). To determine the relative expression levels of Norwalk replicon RNA, β-actin was used as a normalizer and ratios were calculated using the Pfaffl method (14). The expression ratio (Norwalk replicon/β-actin) was calculated as expression ratio = $(E_{\text{Norwalk}})^{\Delta CT,\ \text{Norwalk (CC - TC)}}/(E_{\beta\text{-actin}})^{\Delta CT,\ \beta\text{-actin (CC - TC)}}$, where E_{Norwalk} and $E_{\beta\text{-actin}}$ represent the amplification efficiency ($E = 10^{-1/\text{slope}}$) for the Norwalk replicon and β-actin qRT-PCRs, respectively. Δ*CT*, Norwalk (CC – TC) is the cycle threshold (C_T) of untreated control cells (CC) minus the C_T of cells treated with a compound concentration (TC) obtained with Norwalk replicon primers and probe. Δ*CT*, β-actin (CC – TC) is the C_T of untreated CC minus the C_T of cells treated with a TC obtained with β-actin primers and probe. Efficiency values (E_{Norwalk} and $E_{\beta\text{-actin}}$) were determined for each qRT-PCR. The 50% and 90% effective concentrations (EC_{50} and EC_{90}) were defined as the compound concentration that resulted in 50% and 90% reductions of the relative Norwalk replicon RNA levels.

Clearance rebound assay. HG23 cells were seeded (2.5×10^5 cells) in 25 cm^2 T-flasks. One day later, cell culture medium was replaced for DMEM without G418 containing either a fixed concentration (not cytostatic) of rupintrivir (0.1, 1, 10 μM) or no antiviral compound. When 90% confluent, cells were trypsinized and (i) 2.5×10^5 cells were seeded in a new 25-cm^2 T-flask with the same concentration of compound while (ii) 1.5×10^5 cells from each flask were collected for quantification of replicon/β-actin RNA. In total, cells were passaged five consecutive times in the presence or absence of rupintrivir and in the absence of G418 (clearance phase). For the rebound phase, after clearance passages 2, 4, and 5, cells of each flask were passaged (when 90% confluent) for three consecutive times in the presence of 1.25 mg/ml G418 and in the absence of rupintrivir. Also here, (i) 2.5×10^5 cells were seeded in new 25-cm^2 T-flasks and (ii) 1.5×10^5 cells from each flask was collected for quantification of replicon/β-actin RNA by qRT-PCR. Whereas cultures that have been completely cleared from their replicon will lose the ability to grow in the presence of G418, cultures that still carry the replicon will survive and proliferate again in the presence of the selection marker.

Antiviral and cytotoxicity assay with MNV. The anti-norovirus activity of rupintrivir was determined using an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]-based cytopathic effect (CPE) reduction assay in the MNV/RAW 264.7 model, as previously described (15). Briefly, RAW 264.7 cells were seeded (1×10^4 cells/well) in 96-well plates and infected with MNV (virus strain MNV-1.CW1) at a multiplicity of infection (MOI) of 0.001 in the presence (or absence) of a dilution series of rupintrivir (0.1 to 200 μM). Following 3 days of incubation, cell culture supernatants were collected for quantification of viral RNA load by qRT-PCR with MNV primers and probe as described elsewhere (15). For the MTS reduction assay, 75 μl of MTS-phenazine methosulfate (PMS) solution was added to each well, and the optical density was read at 498 nm (OD_{498}) 2 h later. The EC_{50} was defined as the compound concentration that (i) protected 50% of cells from virus-induced CPE or (ii) reduced MNV RNA copies by 50%. Adverse effects of the drug on the host cell were also assessed by means of the MTS method, by exposing uninfected cells to the same concentrations of rupintrivir for 3 days. The CC_{50} was defined as the compound concentration that reduced the number of viable cells by 50%.

Recombinant production of the norovirus GII 3C-like protease. DNA coding for the norovirus 3C-like protease (GII, strain *Norovirus* Hu/NLV/Dresden174/pUS-NorII/1997/GE) was amplified from the genomic cDNA (kindly provided by J. Rohayem, Riboxx) using the PCR method (forward primer, 5′-AAA ACC ATG GCC CCA CCA AGC ATC TGG TCG AGG-3′; reverse primer, 5′-AAA AGG TAC CTT ATT CAA GTG TAG CCT CCC CCT CAC TC-3′) and inserted into the NcoI and KpnI sites of the pETM10 vector (a kind donation by EMBL, Hamburg). The gene was overexpressed in the *Escherichia coli* strain Rosetta(DE3)pLysS, by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when cells had reached an OD_{600} of 0.6 at 37°C. Subsequently, the cells were further incubated overnight at 20°C, resulting in a protein comprising amino acid residues 1 to 188 of the norovirus 3C-like protease, with the extra sequence MKHHHHHHPM at its N terminus derived from the vector. Cells were harvested by centrifugation and broken by sonication in buffer A (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 0.02% [vol/vol] β-mercaptoethanol [β-ME], 10 mM imidazole). The cell debris was clarified by centrifugation, and the resultant supernatant was applied to an Ni-nitrilotriacetic acid (NTA) resin (Qiagen). After washing the resin with 10 column volumes of buffer A, the protein was eluted with 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.02% β-ME, and 250 mM imidazole. Subsequently, the eluted protein was further purified by gel filtration on HiLoad 16/600 Superdex 75 (GE Healthcare), with 20 mM HEPES (pH 7.4), 200 mM NaCl, and 2 mM dithiothreitol (DTT) as the flowing buffer. The enzyme concentration was determined using the absorption at 280 nm.

***In vitro* fluorescence assay of enzyme activity.** The activity of the enzyme was evaluated by measuring the fluorescent signal (excitation wavelength of 360 nm, emission wavelength of 460 nm) of the dabcyl group of the FRET substrate dabcyl-KDEFELQ ↓ GPALT(D-edans)-amide. Upon cleavage of the substrate at the site indicated by the arrow (↓), quenching of dabcyl fluorescence by the edans group is abolished. The reaction buffer was 50 mM HEPES (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 6 mM DTT, 60% (vol/vol) glycerol. Measurements were performed in 100-μl reaction volumes at 37°C using a BioTek FLx800 microplate fluorescence reader. For the determination of k_{cat} and K_m, substrate concentrations of 1.25, 2.5, 5, 10, 20, and 40 μM and an enzyme concentration of 0.7 μM were chosen. To assess the inhibitory activity of rupintrivir toward the norovirus 3C-like protease, the enzyme was preincubated with various concentrations of rupintrivir (2.5, 5, 10, 20, 40, and 80 μM) for 30 min at 37°C before 20 μM substrate was added. Rupintrivir was dissolved in dimethyl sulfoxide (DMSO), resulting in a total DMSO concentration of less than 1.2% in the assay buffer, an amount that has been shown previously to be well tolerated by norovirus 3C-like proteases (16).

The principle of the kinetic assay and more details have been described elsewhere (17). Briefly, time-dependent progress curves were fitted to a first-order exponential to obtain an observed first-order inhibition rate constant (k_{obs}) (18): $F = (v_0/k_{obs}) \cdot (1 - \exp^{-k_{obs} \cdot t}) + D$.

In this equation, F is the product fluorescence (measured in arbitrary units), v_0 is the initial velocity, t is time, and D is a displacement term accounting for the nonzero emission at the start of data collection. Since in the case of low enzyme activity the measurement of the rate of inactivation, k_3, by an irreversible inhibitor such as rupintrivir tends to be difficult, $k_{\text{obs}}/[I]$, where $[I]$ is the concentration of the inhibitor, was used as an approximation of the pseudo-second-order rate constant.

Analysis of the enzyme kinetics was carried out using the GraphPad Prism software and the nonlinear regression analysis program Origin (OriginLab), in order to obtain the k_{obs} values for enzyme inactivation at three different inhibitor concentrations.

Molecular modeling. Atomic coordinates for rupintrivir were retrieved from PDB 3QZR (complex with the enterovirus 71 3C protease) (19). Atomic coordinates for the Norwalk virus 3C-like protease were

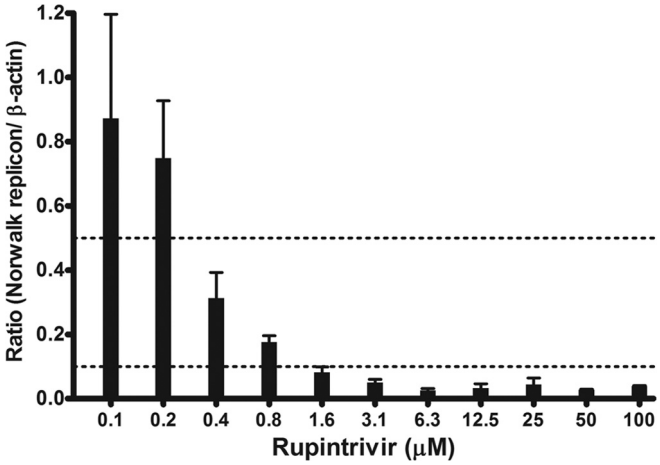

FIG 1 Antiviral activity of rupintrivir against the Norwalk virus replicon, represented by relative genome quantification (Norwalk replicon/β-actin). Results are mean values (±standard errors of the means [SEM]) from 4 independent experiments. The dotted lines represent 50 and 90% reduction of the relative Norwalk replicon RNA levels.

retrieved from the crystal structure of a peptide aldehyde complex (PDB 4IMQ) (20). As this coordinate set lacks residues 123 to 132, these were model-built using the SWISS-MODEL webserver (21). Rupintrivir was manually docked into the substrate-binding cleft of the Norwalk virus $3CL^{pro}$ using Coot (22), based on the knowledge of inhibitor-protein interactions learned from both PDB 4IMQ and PDB 3QZR. Subsequently, the coordinates of the complex were further optimized using the energy minimization function of Yasara (http://www.yasara.org/). The intermolecular contacts were analyzed with Ligplot (23).

Combination studies with 2CMC and T-705. The combined antiviral effects of rupintrivir with 2CMC and T-705 were studied. The effects of drug-drug combinations were evaluated using the method of Prichard and Shipman, through the Mac Synergy II software (24). The theoretical additive effect is calculated from the dose-response curves of individual molecules by the equation $Z = X + Y(1 - X)$, where X and Y represent the inhibition produced by the individual compounds and Z represents the theoretical effect produced by the combination of compounds when additive. The theoretical additive surface is subtracted from the actual experimental surface. When a combination is additive, data points of the calculated surface form a horizontal surface that equals the zero plane. A surface that lies >20% above the zero plane indicates a synergistic effect of the combination; a surface >20% below the zero plane indicates antagonism. For both combinations studied (rupintrivir-2CMC and rupintrivir–T-705), a checkerboard with 3-fold serial dilutions of compounds was studied. These were added to HG23 cells (5,000 cells/well), preseeded 1 day earlier in 96-well plates. Following 72 h of incubation, cell monolayers were collected for quantification of RNA load by qRT-PCR.

RESULTS AND DISCUSSION

Rupintrivir inhibits the replication of the Norwalk replicon and clears human cells from the replicon. The inhibitory effect of rupintrivir on human norovirus replication was assessed by quantification of the levels of Norwalk replicon RNA and of the reference (housekeeping) gene β-actin mRNA, by qRT-PCR. Rupintrivir reduced the levels of Norwalk virus RNA in a dose-dependent manner with an EC_{50} of 0.3 ± 0.1 μM and an EC_{90} of 1.5 ± 0.2 μM (Fig. 1). Such antiviral effect, although 10-fold less pronounced, was also earlier reported (10); differences in the antiviral activity in that and our study may possibly be related to either differences in methodology or in the purity of the compound. No decrease in β-actin levels was observed at any tested concentration, corroborating recently published data (10).

It was next studied whether rupintrivir is able to totally eliminate the norovirus replicon from stably transfected cultures. For that purpose, clearance and rebound experiments were performed. During the clearance phase, a reduction of 3 $\log_{10}$ in Norwalk replicon RNA levels was observed in cultures treated with 10 μM rupintrivir for a single passage; 7 $\log_{10}$ reductions in replicon RNA levels were observed following 4 passages in the presence of 10 μM of the compound (Fig. 2A). Treatment with 1 μM rupintrivir resulted in a decrease in Norwalk RNA of 3 $\log_{10}$ following three passages; at a concentration of 0.1 μM, the reduction of viral RNA levels was 1.6 $\log_{10}$ after five passages. Cultures that have been completely cleared from their replicon will lose in the rebound phase (during which G418 is added) the ability to proliferate, whereas cultures that still carry some replicon will survive and proliferate in the presence of the selection marker. When challenged with G418, cells that had been cultured for two passages in

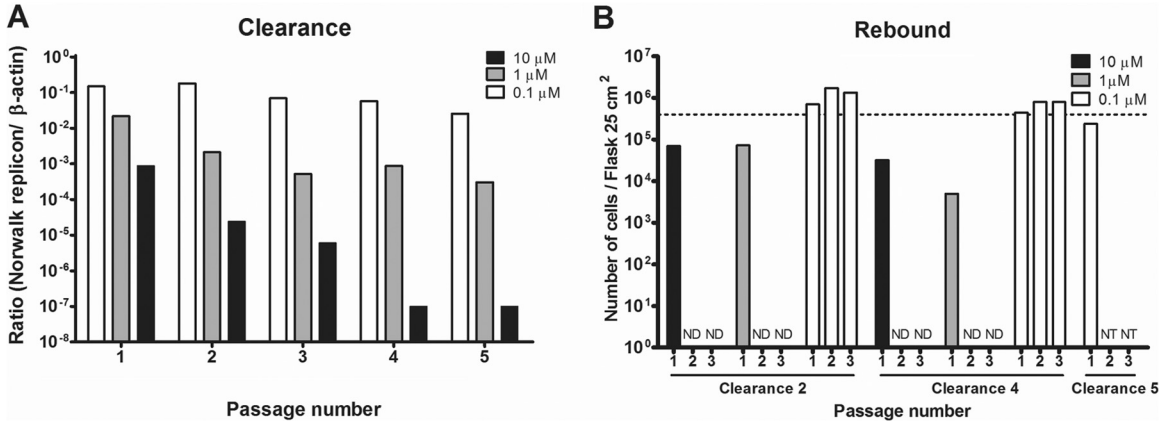

FIG 2 Effect of rupintrivir on the clearance of the Norwalk replicon from HG23 cells. (A) Clearance phase. Cells were treated for five consecutive passages with 3 concentrations of rupintrivir (10, 1, and 0.1 μM) in the absence of selective pressure with G418. (B) Rebound phase. During the rebound passages, rupintrivir was omitted from the culture medium, but cells were again cultured under selective pressure with G418 (1.25 mg/ml). The graph shows the number of cells/25-cm^2 T-flasks for three rebound passages with cells from clearance passages 2, 4, and 5 (ND, not detected; NT, not tested). The dotted line represents the minimum number to subcultivate cells again.

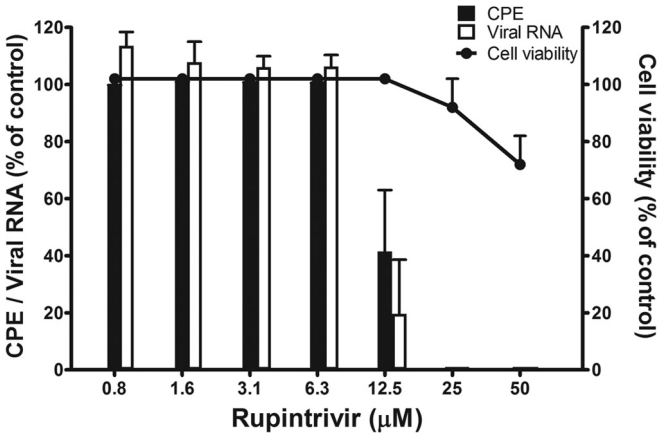

FIG 3 Antiviral activity of rupintrivir against MNV as quantified by (i) virus-induced cytopathic effect (CPE) reduction assay using a colorimetric method (MTS) and (ii) measuring viral RNA levels by means of qRT-PCR. Potential cytotoxic effects were assessed in parallel. Results are mean values (±SEM) from 2 to 4 independent experiments.

the presence of either 1 or 10 μM rupintrivir were no longer able to proliferate in the presence of G418 (Fig. 2 B). Hence, treatment for 9 days with 1 μM rupintrivir is sufficient to clear cells from their replicon. The inability of cells to proliferate once G418 is added again could be due to a low degree of fitness of replicon RNA following treatment with rupintrivir. Population sequencing would have been necessary to assess if that was indeed the case. At lower concentrations (0.1 μM), cultures were not cleared, even after five passages in the presence of the compound. It has been demonstrated by selecting for drug-resistant variants that rupintrivir indeed targets the viral 3C protease. Resistance developed slowly (unless what is observed for HCV protease inhibitors, for example) with accumulation of multiple mutations in the rhinovirus protease. The efficient clearance of the Norwalk replicon from the host cell by rupintrivir suggests that resistance against noroviruses may also develop slowly. Indeed, during HCV replicon clearance experiments with HCV protease inhibitors, resistance developed readily, and clearance was, as a result, virtually impossible (25).

The anti-norovirus activity of rupintrivir is extended to MNV. To study whether rupintrivir exerts cross-genotypic norovirus activity, we next assessed whether the drug is able to inhibit the replication of MNV, a genogroup V norovirus. MNV has been used as a surrogate for human noroviruses (given that these are not cultivable) and shares many of their structural and replicative features (26, 27). Rupintrivir inhibited in a dose-dependent manner MNV-induced CPE (EC_{50} = 13 ± 2 μM) (Fig. 3) (although no activity was reported in another study [10]). We confirmed this anti-norovirus activity by assessing the effect of rupintrivir on MNV RNA synthesis; rupintrivir inhibited the production of MNV RNA, also in a dose-dependent manner (EC_{50} = 10 ± 1 μM) (Fig. 3). Furthermore, levels of MNV RNA were reduced by >2 $\log_{10}$ when cells were treated with >12.5 μM rupintrivir. This inhibitory effect was, however, less pronounced than against the Norwalk replicon; little adverse effect on the host cell was noted (CC_{50} = 77 ± 7 μM).

Rupintrivir has an inhibitory effect on a GII norovirus 3C-like protease. The effect of rupintrivir on the recombinant 3C-like protease of a GII norovirus (strain Hu/NLV/Dresden174/pUS-NorII/1997/GE) was next assessed. This enzyme has about 63% amino acid sequence identity to the 3C-like protease of the GI Norwalk virus. The GII protease cleaved the FRET substrate dabcyl-KDEFELQ ↓ GPALT(D-edans)-amide (corresponding to the NS3/NS4 site) with k_{cat} = 3.25 ± 1.03 min^{-1}, K_m = 188.2 ± 69.8 μM, and k_{cat}/K_m = 17.27 × 10^3 M^{-1} min^{-1}. This activity is low, albeit somewhat higher than that described by Chang et al. (16), for 3C-like proteases of various other norovirus strains and for other substrates. In our assay, the addition of 60% glycerol was necessary to observe enzyme activity, as previously described (16). This excessive glycerol concentration led to a high viscosity of the assay buffer solution and is the likely cause of the large standard deviations in the kinetic constants. The errors introduced by the high viscosity of the assay buffer were estimated to be larger than those due to inner-filter effects in the fluorescence measurements in the 96-well plate (28, 29); hence, no attempts were made to correct for the latter.

To characterize the inhibitory activity of an irreversible inhibitor such as rupintrivir, which binds covalently to the catalytic cysteine of the protease, the rate of inactivation, k_3, should be determined in addition to K_i. This proved difficult, though, because of the relatively low enzyme activity, as mentioned above. Therefore, we determined instead the ratio $k_{obs}/[I]$ (see Materials and Methods). Using this approach, rupintrivir was found to inhibit the norovirus 3C-like protease with $k_{obs}/[I]$ = 12.6 ± 3 M^{-1} s^{-1}, indicating a weak but significant inhibitory activity of the compound.

The fact that rupintrivir is active against noroviruses belonging to genogroup I (Norwalk virus; EC_{50} = 0.32 ± 0.13 μM; EC_{90} = 1.5 ± 0.3 μM) and genogroup V (MNV; EC_{50} = 13 ± 5 μM by CPE reduction and 10 ± 1.6 μM by qRT-PCR), as well as against the recombinant 3C-like protease of a GII norovirus ($k_{obs}/[I]$ = 12.6 ± 3 M^{-1} s^{-1}), suggests that the drug exerts cross-genotypic anti-norovirus activity and will thus most likely be effective against the clinically relevant human norovirus strains (values given are means ± standard deviations [SD] from four independent experiments, except for MNV qRT-PCR, where two independent experiments were performed).

Molecular modeling shows fitting of rupintrivir in the Norwalk virus protease-active site. As rupintrivir was developed as an inhibitor of the 3C protease of human rhinoviruses (7, 8), we assessed its potential of inhibiting the 3C-like protease of Norwalk virus by molecular modeling, based on the crystal structure of the free enzyme (11). The active site of the Norwalk virus protease bears similarities to that of the enterovirus 3C proteases (30, 31); the catalytic triad is formed by residues Cys139, His30, and Glu54 (Fig. 4). The rupintrivir molecule can be placed in the substrate-binding pocket of the protease (Fig. 4A), with the covalent bond formed between the Cβ atom of its vinylogous ester moiety and the nucleophilic Cys139 serving as one of the anchor points. Another anchor point is the hydrogen-bonding interaction between the lactam oxygen of the P1 substituent and conserved His157 in the S1 pocket (Fig. 4B). The fluorobenzyl substituent in the P2 position of the inhibitor fits smoothly into the enzyme's S2 pocket, the walls of which are formed by residues Glu54, Ile109, and Gln110. According to our model, the main-chain amides and carbonyl oxygens of rupintrivir can make several hydrogen bonds with the target protease. Judging from this interaction model, the

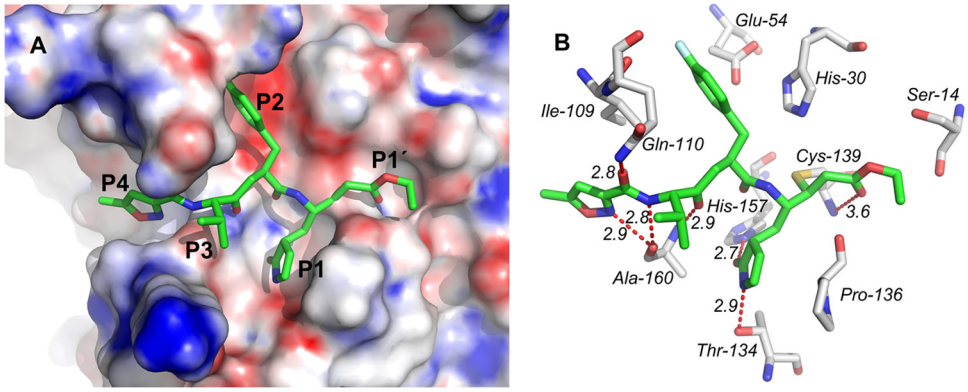

FIG 4 Structural model of the complex between rupintrivir and the 3C-like protease of Norwalk virus. (A) Binding of rupintrivir to the substrate-binding site of Norwalk virus $3CL^{pro}$. The electrostatic potential at the surface of the protein is displayed; red, negative potential; blue, positive potential. (B) Details of the proposed interactions between rupintrivir (green sticks, with oxygen in red and nitrogen in blue) and the Norwalk virus 3C-like protease (gray sticks, with oxygen in red and nitrogen in blue). Proposed hydrogen bonds are indicated as red dashed lines, with distances in Å. The orientation is the same as that for panel A.

observed inhibitory activity of rupintrivir against the norovirus 3C-like protease can be explained.

Combination of rupintrivir with polymerase inhibitors 2CMC and T-705 has an additive antiviral effect. We demonstrated recently that 2′-*C*-methylcytidine (2CMC) and favipiravir (T-705) inhibit *in vitro* norovirus replication (15, 32); both molecules are inhibitors of viral RNA-dependent RNA polymerases (RdRp). We assessed the combined antiviral effects of rupintrivir with 2CMC and T-705 in the Norwalk replicon system. 2CMC inhibited the replication of the Norwalk replicon with an EC_{50} of 18 ± 4 μM (33), while T-705 had modest activity (EC_{50} of 287 ± 98 μM [our unpublished data]). The mean volume of synergy/antagonism observed was 29 μM^2% (17 to 41 μM^2%, 95% confidence interval) for the 2CMC-rupintrivir combination and 15 μM^2% (0 to 30 μM^2%, 95% confidence interval) for the T-705-rupintrivir combination. Hence, both combinations resulted in an overall additive antiviral activity (Fig. 5), which is expected of compounds whose mechanism of action is distinct and that do not interfere with each other's metabolism or mechanism of action.

Rupintrivir was initially developed as an intranasal product to treat rhinovirus infections (7, 8). Given its poor oral bioavailability, the molecule does not have the ideal pharmacokinetics profile for a prophylactic approach to prevent norovirus infections. Noroviruses are typically involved in extensive outbreaks in semi-closed environments, where person-to-person transmission is usually the main route of transmission. Yet, noroviruses often cause prolonged and severe and potentially even life-threatening infections in elderly and immunocompromised individuals (34, 35). Rupintrivir could thus be relevant as a starting point for further drug design activities toward a molecule with sufficient potency and pharmaceutical properties to be useful in the treatment of such risk populations.

In conclusion, we here show that the protease inhibitor rupintrivir exerts antiviral activity against two different genogroups of

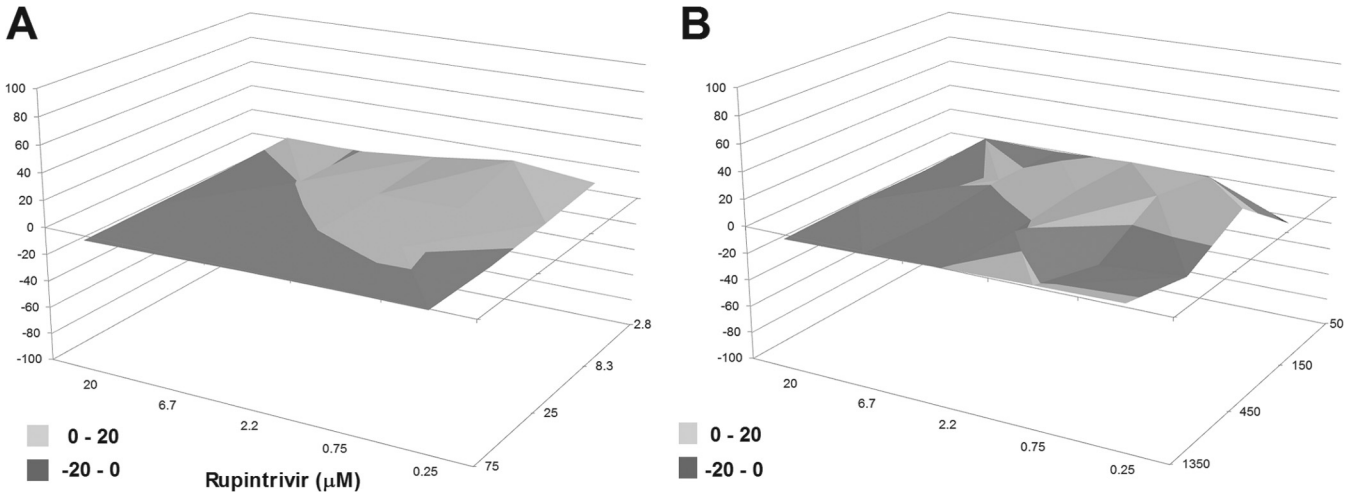

FIG 5 Antiviral effect of the combination of either rupintrivir and 2CMC (A) or rupintrivir and T-705 (B). Values in the zero plane indicate additive activity; a surface below the zero plane indicates antagonism; over 20% above the plane indicates synergism. Results are mean values from 2 independent experiments.

norovirus and inhibits the protease of yet another genogroup. It is therefore very likely that this molecule is able to inhibit the clinically relevant GI and GII noroviruses (which cannot be cultured). Our findings suggest that the design of protease inhibitors of noroviruses could be a successful approach for the treatment/prevention of norovirus infections.

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